Title: A New and Simple Method for Spinal Cord Injury Induction in Mice

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Plain language summary

Spinal cord injury (SCI) is a medical problem which can lead to permanent motor and sensory dysfunction. Traffic accidents, falls and violence are the most frequent causes of SCI which often affect young individuals. Patients and even their families may encounter other problems including reduction in life quality, psychological burden and enormous medical cost. Despite scientific and technological advances, there is no effective treatment for it. Therefore, animal models are useful for recognizing damage mechanisms as well as evaluating novel treatment strategies. All SCI research centers require an economical and reproducible device with no requirement of complex surgical procedures by experienced surgeons to minimize variations in damage. In this study, a simple, economical and reproducible novel instrument for SCI induction was introduced. The instrument consists of various sections, including a body part, an immobilization piece and a bar-shaped weight. Eight-gram weight for 5, 10 or 15 minutes was used to inflict injury to spinal cord. Behavioral and tissue studies indicated that SCI can be induced in rodents in different severity without any excess accessory elements. This instrument can be used in future based investigations for SCI studies including tissue engineering, stem cell therapy and drugs delivery to access an effective treatment.

Highlights

- A simple and precise method has been introduced for creating spinal cord injury (SCI) in mice by using a novel device.
- The main parts of the device are a body part, an immobilization piece and a bar-shaped weight.
- Assessment of locomotor activity, tissue damage and, macrophage infiltration confirmed the capability of the new SCI method.
- Reduction of adverse spinal movements and working without any need for accessory elements are the key points of this new animal model of SCI.
Abstract

Introduction: Spinal cord injury (SCI) is a devastating disease with poor clinical outcomes. Animal models provide great opportunities to expand our horizons in identifying SCI pathophysiological mechanisms and subsequently introducing effective treatment strategies. The present study precisely introduces a new murine contusion model.

Methods: A simple, economical and reproducible novel instrument was designed which consists of various sections, including a body part, an immobilization piece and a bar-shaped weight. The injury was inflicted to the spinal cord using an eight-gram weight for 5, 10 or 15 minutes after laminectomy at the T9 level in male C57BL/6 mice. Motor function, cavity formation, cell injury and macrophage infiltration were evaluated 28 days' post injury.

Results: The newly designed instrument minimized adverse spinal movement during injury induction. Moreover, no additional devices, such as a stereotaxic apparatus, was required to stabilize the animals during surgical procedure. Locomotor activity was deteriorated after injury. Furthermore, tissue damage and cell injury were exacerbated by increasing the duration of weight exertion. In addition, macrophage infiltration around the injured tissue was observed 28 days’ post injury.

Conclusion: This novel apparatus could induce a controllable SCI with a clear cavity formation in mice. No accessory elements are needed, besides the main equipment, and it can be used in future SCI studies.

Keywords: Spinal cord injury, Animal models, Mice
1. Introduction

Spinal cord injury (SCI) is a major medical problem with severe disability and high mortality rates. Because of the important role of the spinal cord as the connecting pathway for most neural control mechanisms, injuries here lead to dysfunction of motor, sensory and autonomic systems (Khan, Havey, Sayers, Patwardhan, & King, 1999; Yip & Malaspina, 2012). It is clear that the SCI is created not only by primary mechanical damage but also through secondary mechanisms. Despite all of the scientific and technological advances, there is still no definitive treatment available for this progressive neurological disease. In this way, animal models can be helpful in terms of understanding the molecular pathways involved in secondary damage as well as evaluating novel treatment strategies (Cheriyan et al., 2014; Onifer, Rabchevsky, & Scheff, 2007).

Animal models of SCI are classified into transection, contusion and compression models. Although transection is rarely seen clinically, contusion and compression injuries are more common (McDonough & Martínez-Cerdeño, 2012). Transection SCI models include all forms of spinal cord laceration. Two common transection models consist of complete transection, in which the distal part of the whole spinal cord is disconnected from the more proximal parts and hemisection, in which half of the spinal cord is severed. Complete severance of axons and the formation of a glial scar occur in the transected area (Breyer et al., 2017; Onifer et al., 2007; Xu, Guénard, Kleitman, & Bunge, 1995).

In contusion injuries, a weight drop method or a dedicated apparatus such as the New York University (NYU), Ohio State University (OSU) or Infinite horizon (IH) impactor are used (Cheriyan et al., 2014). The severity of the injury depends on the weight or the height from which the
impactor tip is dropped. This experimental model results in cavity formation within the spinal cord tissue while the glial limitans remains intact (McDonough & Martínez-Cerdeño, 2012). Application of the available devices for SCI induction is limited by their expensive costs and the requirement of complex surgical procedures by experienced surgeons to minimize variations in damage (Farooque, 2000; Kwon, Oxland, & Tetzlaff, 2002).

The compression injury can be created by calibrated forceps (McDonough, Monterrubio, Ariza, & Martínez-Cerdeño, 2015; Plemel et al., 2008), aneurysm clips (Rivlin & Tator, 1978), or putting a weight directly on to the spinal cord (Wu, Shibuya, Miyamoto, Itano, & Yamamoto, 2005). Compression is similar to contusion in pathological features and neurological impairment. Severity of an injury can be increased by enhancement of compression power, aneurysm clips capable of applying greater pressure, or increasing the duration of the spinal cord compression (Cheriyan et al., 2014). Calibrated forceps are a simple, inexpensive, consistent and highly reproducible method. However, recording the amount of force is not possible.

In clip compression the force velocity and compression extent cannot be recorded (Abdullahi, Annuar, Mohamad, Aziz, & Sanusi, 2017). In the weight-drop compression model the spinal cord is only briefly impacted, and thus, it does not reproduce the effect of prolonged cord compression. Considering these limitations, improvement of the current SCI animal models is required.

The present study introduces a feasible method for creating SCI in rodents using a simple, inexpensive and well-designed novel instrument. For calibration several factors were evaluated
including: quantitative functional outcomes, size of tissue damage, rate of cell injury and macrophage infiltration.

2. Methods

First, a new device was designed and built to induce rodents SCI. Then to evaluate the effectiveness of our new device, twenty two adult male BALB/C mice between 26g to 30g of weight were used to develop compression SCI. Mice were randomly divided into four groups including control (CNT) group, which underwent laminectomy without cord injury and SCI groups in which damage was induced with an 8-gram weight applied for 5, 10 or 15 min. Subsequently, behavioral and histological assessments were performed. The animals were Purchased from the Center of Comparative and Experimental Medicine at the Shiraz University of Medical Science in Iran and were housed in animal lab of school of advanced medical sciences and technologies accordance with standard conditions of ambient temperature (22 ± 2°C), 12:12 h light-dark cycle, and free access to water and food. All procedures were done in accordance with the institutional guidelines of Shiraz University of Medical Sciences for animal care and use.

2.1. SCI apparatus

As indicated in Figure 1, the SCI induction device consists of various sections, including a body part, an immobilization piece, and a bar-shaped weight. On each side of the body, a three-legged stand is fixed on a 35 x 25cm long plate to increase the stability of the device at the top of the instrument, a 15 x 3.5 cm-long rectangular piece with a hole in middle. The immobilization piece is similar to the above-mentioned rectangle plus four prong-like pieces located below. The immobilization piece can be moved up and down in a vertical line up to 2.5 cm. This part of the device is used to fix the spine and prevent its probable vertical and horizontal movements during
the SCI procedure. The weight is an 8-gram stainless steel metal rod with 27 cm length and a 1 x 2 mm tip. The tip is curved to fit completely on the surface of the spinal cord. When used, the rod is passed through the holes in the middle of the upper part and the immobilization piece. A 10 x 32 cm plate is considered the animal bed, serving the purpose of keeping the animal under the device.

2.2. SCI induction

Before surgery, all surgical tools were sterilized to prevent any possible contamination. Firstly, the animal was weighed and then anesthetized by ketamine (90 mg/kg) and xylazine (10 mg/kg). Then the mouse was placed on a surgical table previously sterilized by 70% alcohol. A heat pad was used to maintain body temperature in order to ensure proper anesthesia, and the animal was checked for reflexes using the toe pinch. The dorsal side was covered by surgical drape with an aperture above the surgical area. To prevent ocular damage, eye ointment was applied on the cornea. After shaving the dorsal area around the incision site, the skin was cleaned with Betadine solution and a longitudinal incision was made using scalpel blade to expose muscles and vertebral column at the T8–T12. In order to keep the incision widely open, the skin was held back with retractor and muscles and fascia along the spinal cord were cut. The tissue was detached from the spinus process and posterior lamina of T9. Without any damage or pressure to the spinal cord, connections between upper and lower vertebrae were slowly drilled and the T9 posterior lamina was carefully removed with forceps to expose spinal cord. Subsequently, the mouse was kept on the animal bed and transferred under the SCI device. After that, the immobilization piece was completely fixed on the upper surface of the spine so that the middle hole was placed adjacent to the exposed spinal cord. Then, the weight was passed through the holes in the middle
of the upper part and the immobilization piece and placed on the top of the exposed spinal cord for a defined period of time (figure 2). After setting the weight on the spinal cord, sterile saline was applied to regain and keep homeostasis. The muscular layer and skin over the spinal cord were carefully sutured. 1ml of saline was injected subcutaneously in order to prevent dehydration. When the animals were awakened, 1.5 mg/kg tramadol was given which was repeated every 12 hours for 48 hours. In addition, 4 mg/kg gentamicin was administrated once every day for 3 days. Mice were separately kept in cages in an environment with appropriate temperature and a 12-hour light/dark cycle for 4 weeks after surgery. Mice were weighed on the first day after surgery and then at the end of each week. Bladder was manually expressed three times a day until bladder reflex occurred. In the first days, animals were monitored for infection (bloody or cloudy urine), weight loss and mobility.

2.3. Behavioral assessment

Basso Mouse Scale (BMS) locomotor scoring system, a sensitive, valid and reliable pre-clinical screening tool for evaluation of motor function recovery in mice which ranges from 0 (complete paralysis) to 9 points (completely normal), was used for behavioral assessment (Basso et al., 2006). Animals were placed on a flat surface and hindlimb motor function was scored by two independent, blind, observers one day after surgery and then once a week for 4 weeks.

2.4. Tissue preparation

At the end of the fourth week, the animals were anaesthetized and sacrificed by intracardial perfusion with 0.9% cold normal saline followed by 1% paraformaldehyde (PFA) in 0.1 M PBS with pH of 7.4. After opening dorsal skin and breaking the ribs, the spinal column related to the damaged area was removed, excess tissue around it was completely detached and it was placed
in 1% PFA for 24 hrs at 4 °C. Subsequent to post-fixation, the spinal cord was removed from the spinal column by scissors and forceps. For histological studies, the tissues were dehydrated in 70, 80, 96 and 100% alcohol respectively, cleared in xylene, and impregnated in paraffin wax.

2.5. **Nissl staining and measuring the lesion volume**

To assess the volume of the lesion cavity, a series of 8 μm sections with an interval of 80 μm were prepared and stained with 1% cresyl violet (Nissl staining) as described (Aligholi et al., 2014). The prepared slides were studied under a light microscope and photos were taken. Infinity analyze software was used to analyze the photos and the lesion volume was calculated by the following formula: 0.5D(A1+An)+D(A2+A+... +An-1), where A is the area of the cavity and D is the distance between sections (Guo et al., 2009).

2.6. **Evaluation of cell injury**

The Nisle-stained sections were used to evaluate cell injury. The cells with shrunken features, and corkscrew-like processes, were considered dark cells. The percentage of dark cells per defined area was calculated by the following formula using infinity software.

The percentage of dark cells= (the number of dark cells/total number of cells) x 100 (Ooigawa et al., 2006).

2.7. **Immunofluorescence assay**

The presence of macrophages around the injury site was investigated by measuring the expression of the CD68 (ED1) as macrophage marker using immunofluorescence assay. Sections were incubated overnight at 4 °C with rabbit anti-CD68 primary antibody (1/200, abcam, Germany). Goat anti-rabbit secondary antibodies (1/600, FITC, abcam, Germany) were applied
for 1 hr at room temperature. Slides were visualized using an Olympus microscope and pictures were taken. Immunofluorescence was quantified by image j software (Jafarian et al., 2019).

2.8. Statistical analyses

All data was indicated as mean ± standard error of the mean. Statistical analyses were calculated using Statistical Package for Social Sciences (SPSS) software (version 23, Chicago, USA). For the assessment of changes over different groups, one-way ANOVA test followed by Pair-wise comparison of the groups using post-hoc testing with LSD correction was used. A repeated measure ANOVA with a Greenhouse-Geisser correction was used to assess changes over time. In all analyses, the significance for the overall group effect and individual pairwise comparisons was defined as p<0.05.

3. Results

3.1. Effect of SCI on locomotor behavior

As illustrated in figure 3A, a repeated measures ANOVA with a Greenhouse-Geisser correction determined that BMS mean score differed significantly between time points (F(7,44)11.8)=11,P<0.01. Placing the weight on spinal cord for 5 minutes could cause mild hindlimb paralysis at day 1 post-injury. BMS score in this group was decreased to 7±0.4 at day 1 and recovered to 9±0 at day 28. BMS score in 5min group was significantly higher at days 1, 7, 14, 21 and 28 post-injury compared to that of 10 and 15min groups (P<0.01). On the other hand, complete hindlimb paralysis was achieved by placing the weight on spinal cord for 10 and 15 min at day 1 post injury. The recovery process was accelerated in the 10min group from 7 day post-
injury, so that the BMS score of animals in the 10min group was significantly different from that of the 15min group in days 21 and 28 post-injury (P<0.01).

3.2. Effect of SCI on body weight

The body weights of the mice were also evaluated at days 1, 7, 14, 21 and 28 post-injury (F(6,36)=14, P<0.05). On the day after surgery all mice showed weight loss, which continued during the first week after surgery in 15min group. After that, weight-gaining process was observed in all groups until 28 days after surgery. However, the body weights of animals in 5 and 10min groups were considerably higher than that of animals in 15min groups in days 21 and 28 post-injury (P<0.05 Figure 3B).

3.3. Effect of SCI on lesion volume

The size of cavity created by SCI was measured using Nissl-stained sections. The results showed that this parameter increased as the time of SCI induction became longer (figure 4). The size of cavity in the spinal cord of animals received weight for 10 min (0.25±0.02) was significantly more than that of animals in the 5min group (0.013±0.0014, P<0.001). In addition, the size of injury in the 15min group (0.4±0.02) was considerably higher than that of the 5 and 10min groups (P<0.001).

3.4. Effect of SCI on cell injury

The percentage of dark neurons as an index of cell injury was evaluated by Nissl staining (figure 5). The results showed that the existence of dark neurons around the injury site in the 10min group (32±0.8) was significantly more compared to the 5min group (18.26±0.6, P<0.001). Moreover, this index in 15min group (52±1.2) significantly increased compared to the 5min and
10min groups (P<0.001). The percentage of dark cells in the 5min, 10min and 15min groups was remarkably higher than that of CNT group (P<0.001).

3.5. Effect of SCI on macrophage infiltration

The distribution of ED1 (macrophage marker) was studied by immunofluorescence assay. The results revealed that the distribution of macrophages was significantly higher in the 5min, 10min and 15min groups than that of the CNT group (P<0.001). In addition, macrophage infiltration showed an increasing trend from the 5min group to the 15min group, however, there was no statistical significance among the groups (figure 6).

4. Discussion

The present study introduced a novel, simple, reproducible and economical method to produce a mice model of SCI. A worsening trend in locomotor activity scores as well as an increasing trend in tissue damage and cell injury indices, following an increase in injury induction time, confirmed the efficiency of the method.

Different models and devices have already been introduced for SCI induction (McDonough & Martínez-Cerdeño, 2012; Plemel et al., 2008; Rivlin & Tator, 1978). One of the most commonly used models is the weight-drop technique that was established by Allen in 1911 (Allen, 1911). Thereafter, efforts were made to create more precise controllable models that led to the creation of some complex and expensive devices (Deep et al., 2015). Here, we designed and introduced a simple instrument that can be made without any complexity. One of the advantages of this new device is that one can induce SCI independently of any additional devices, like the stereotaxic apparatus. In this sense, the immobilization part of the device helps us to induce SCI with the least adverse spinal movement. Moreover, not only can the induction time be changed, but so
can the weight, to create SCI models with different intensities. Furthermore, the device is applicable for induction of SCI in other rodents like rats.

This model of SCI affects the motor function of hindlimb as well as the function of urinary system. After manual massage of bladder, the bladder reflex generally returned during the first week post injury. Using analgesic drugs in the first days post SCI helps the animal to tolerate pain and facilitate seeking for water and food. Behavioral assessment revealed that hindlimb motor dysfunction correlated with the compression time. In other words, BMS scores reduced when compression time increased. Based on the results of BMS test, 5 min compression created a weak SCI that was completely recovered after 28 days. Although, 10 min and 15 min compression created a moderate to severe SCI respectively which remained for 28 days.

Whole body weight as a general indication of health was measured in this study. We observed weight loss that normally occur after SCI (Landry, Frenette, & Guertin, 2004). Almost all mice had 1-2 gr body weight loss one day after injury due to the direct effect of surgery. In the 5min group, mice compensated for this weight loss quickly during the first week after injury because of how weak the injury was and how they were getting enough water and food. In 10min group, weight gaining was slower but the mice compensated for their weight loss around the second week and then followed the natural process of weight gaining. Although the weight gaining was very slow in the 15min group, there was no excess weight loss after the first week post injury.

The formation of a cavity after SCI is one of the main pathologic futures that occurs following secondary injury (Ahuja et al., 2017; Chen et al., 2016; Osaka et al., 2010). During the first hours after primary SCI, the secondary injury starts through several mechanisms including an increase
in intracellular calcium ion, activation of proteases, activation of autophagy, secretion of inflammatory elements, activation of microglia, migration of macrophages and astrogliosis. Although the first aim of these mechanisms is supporting the damaged tissue, they act as a double-edged sword. After activation of the secondary mechanism and elimination of the damaged tissue, a cavity is formed during the early weeks following SCI. Thus, formation of a cavity is one of the necessities in properly establishing animal models of SCI (Deep et al., 2015; Sroga, Jones, Kigerl, McGaughy, & Popovich, 2003; Surey, Berry, Logan, Bicknell, & Ahmed, 2014). In this study we observed cavity formation in all three groups. The cavity was very small in the 5min group but by increasing the induction time, the cavity became larger so that they were more obvious in the 10 and 15mine groups. It is worth pointing out that cavity formation in animal models of SCI is an interesting option for scientists’ working on neuro-regeneration using tissue engineering.

In the present study, macrophage infiltration was evaluated after SCI. In this sense, the presence of macrophages around the damaged tissue was observed 4 weeks after SCI. Spinal cord injury is associated with macrophage activation however the effect of this phenomenon remains controversial. As previous reports, the activation of these cells was maximal between 3 and 7 days post injury (Carlson, Parrish, Springer, Doty, & Dossett, 1998; Zhang, Krebs, & Guth, 1997) but Wu et al demonstrated that they were observed at 4 weeks and reduced around 12 weeks after injury in rat (Wu, Miyamoto, et al., 2005). The clear issue is that by using our model of SCI, the activation of macrophages can be evaluated up to 4 weeks post injury in mice.
In conclusion, by using this new and simple apparatus, a controllable SCI can be induced in rodents without any excess accessory elements. Creation of compression injury with a clear cavity can be helpful in future tissue engineering-based investigations for SCI.

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References


Figure 1. SCI apparatus. The device used for creation of SCI in rodents is illustrated in A. The injury is made by a bar-shaped weight (blue arrow). The immobilization piece (B) can move up and down in a vertical line (yellow arrow). Four prong-like pieces (C, green arrow) are located below the immobilization piece for fixing the spine. The tip of the weight is curved (D, pink arrow) similar to the surface of the spinal cord. The device works without any excess accessory.
Figure 2. Induction of SCI by the new device. After laminectomy and exposing spinal cord (A), the animal was transferred under the SCI device (B) and the spine was fixed by prong-like pieces of immobilization piece (C).
Figure 3. Locomotor behavior and body weight following SCI. A: The BMS scores were calculated every week to evaluate the locomotor behavior of mice after SCI. B: The body weight of the animals was assessed weekly in the different groups. $: P<0.01$ VS other groups. @: $P<0.001$ VS 10min and 15min groups. &: $P<0.01$ VS 10min group. *: $P<0.05$ VS 10min and 15min groups. #: $P<0.05$ VS 5min and 10min groups.
Figure 4. Lesion volume following SCI. By using Nissl-stained sections, the size of injury induced by the apparatus in the CNT (A), 5min (B), 10min (C) and 15min (D) groups was calculated and quantitative results is illustrated in (E). *: P<0.001 VS 5min and CNT groups. #: P<0.001 VS 10min group. Scale bar represents 200 μm.

Figure 5. Cell injury following SCI. The percentage of dark cells (white arrow) per area was considered as an index of cell injury in the CNT (A), 5min (B), 10min (C) and 15min (D) groups. The quantitative data is shown in (E). *: P<0.001 VS other groups. Scale bar represents 50 μm.
Figure 6. Macrophage infiltration following SCI. The percentage of ED1-positive cells (white arrow) per area was considered as an index of macrophage infiltration in the CNT (A), 5min (B), 10min (C) and 15min (D) groups. (E) indicates the quantitative data. *: P<0.001 VS CNT group. Scale bar represents 100 μm.